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Identification of a Polymorphically Expressed Member of the Human Cytochrome P-450III Family

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SUMMARY

The human liver cytochrome P-450III family contains two highly related forms of cytochrome P-450 (P450), HLp and P450NF, that are expressed in the adult and a form, HLp2, that is expressed in the fetus. Immunoblot analyses of 46 liver specimens developed with an anti-HLp antibody demonstrated that, in addition to HLp, 11 specimens contained a previously undetected higher molecular weight protein (termed HLp3). The expression of HLp3 did not correlate with the age, gender, smoking habits, or drug history of the patients. This protein was purified and found to be a P450 with a molecular weight of 52,000. Ouchterlony analyses using a polyclonal anti-HLp antibody yielded lines of partial identity between HLp3 and both HLp and HLp2. In addition, structural comparisons between these three proteins, including amino-terminal amino acid analyses and peptide mapping, indicated that HLp3, HLp2, and HLp are highly related but distinct proteins. In reconstituted systems, HLp and HLp3 were found to hydroxylate testosterone at the 6β and 2β positions. In microsomes, the rates of hydroxylation of testosterone at the 2β, 6α, 6β, 15β, 16β, and 18 positions and the formation of an unknown were found to correlate with the levels of total HLp-related protein and these activities were inhibited by anti-HLp antibodies. In conclusion, our data demonstrate that HLp3 is a member of the human P450III family and is polymorphically expressed.

The P450s are a superfamily of hemoproteins that are responsible for the oxidative metabolism of a large number of compounds including xenobiotics, such as drugs and pesticides, and endobiotics, such as prostaglandins and steroids (1–3). Recently, a great deal of progress has been made in the identification and characterization of human liver P450s with immunologic, purification, and recombinant DNA techniques combining to characterize at least 12 different human liver P450s (4–12). Three of these forms of P450, P450NF, HLp, and HLp2, comprise the human steroid-inducible P450III family (3–6, 13). Two members of this family, P450NF and HLp, are highly related, differing by only 11 of 503 amino acids (13), and together account for up to 30% of the total P450 present in the liver of the adult (4–6). The third member, HLp2, represents the majority of the P450 present in the fetal liver (6). In addition, the levels of HLp (P450NF)[1] have been shown to be induced in humans by administration of macrolide antibiotics (e.g., rifampicin and triacetyloleandomycin) (4, 14) and glucocorticoids (e.g., dexamethasone) (4).

The human liver P450III family is clinically very important, because the members of this family have been shown to be responsible for the metabolism of a large number of drugs. For example, HLp or P450NF has been shown to be responsible for the metabolism of erythromycin, benzphetamine, aldrin, quinidine, cyclosporin, and dihydropyridine calcium channel blockers such as nifedipine (4, 5, 14, 15). This family of P450s also appears to be responsible for the 6β-hydroxylation of steroids, including testosterone, androstenedione, progesterone, and cortisol (16, 17). Finally, unlike the fetus of the rodent, the human fetus is capable of metabolizing a large number of compounds and HLp2 has been shown to be the major form of P450 expressed in fetal liver (6). Thus, the P450III family of P450s plays a crucial role in xenobiotic and endobiotic metabolism by the liver throughout human development.

Analyses of the total genomic DNA related to HLp suggest that there are at least five genes in the human P450III family (18). However, the number of these genes that are actually expressed is not known. We report here that, in addition to HLp, a protein (termed HLp3) that is related to the P450III family is expressed in 11 of 46 human liver specimens. Moreover, the levels of HLp3 do not appear to correlate with the

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[1] The exact relationship between HLp and P450NF, with respect to their catalytic properties, is currently not understood. However, the monoclonal antibody used in this report recognizes both HLp (4, 18) and P450NF (5, 28). Thus, the levels of HLp-related protein determined by immunoquantification studies with this antibody would reflect the combined level of HLp and P450NF, if they are indeed different proteins. In addition, because the antibody preparations used in immunoinhibition studies also appear to react with both HLp and P450NF, it is not currently possible to determine whether catalytic differences exist between these two forms.

ABBREVIATIONS: P450, cytochrome P-450; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

HLp levels or with the ages, smoking habits, genders, or drug histories of the patients. In addition, we purified HLp3 and through structural, functional, and immunochemical studies demonstrated that, although it is highly related to the other members of the human P450III family (HLp, P450NF, and HLp2), it is clearly a distinct form of P450. Finally, using a highly sensitive HPLC technique, we find that the members of the P450III family catalyze several stereospecific hydroxylations of testosterone that have not been previously observed with human liver microsomes.

Experimental Procedures

Materials. Tergitol NP-10, L-dilauroylphosphatidylcholine, testosterone, 11β-testosterone, α-chymotrypsin (type I-S), and *Staphylococcus aureus* V8 protease (type XVII) were purchased from Sigma Chemical Company (St. Louis, MO). Sepharose 4B-CL was obtained from Pharmacia (Piscataway, NJ). Aldrich (Milwaukee, WI) was the source of 1,8-diaminooctane. Nitrocellulose, secondary antibodies, SDS-polyacrylamide gel electrophoresis reagents, silver stain kits, and hydroxylapatite (Bio-Gel HTP) were purchased from Bio-Rad (Richmond, CA). Whatman DE-51, DE-52, DE-53, and CM-52 celluloses were purchased from Bodman (Doraville, GA).

Liver specimens. The human liver specimens were obtained at surgery in accordance with protocols approved by the Committee for the Conduct of Human Research at the institution (The Medical College of Virginia, Richmond, VA; The Medical College of Wisconsin, Milwaukee, WI; or The University of Michigan, Ann Arbor, MI) at which they were received. All patients had normal bilirubin and transaminase levels. Patient code numbers refer to individual liver specimens obtained at The Medical College of Virginia, with numbers 1 through 23 having appeared in other studies (4, 7–9). Code numbers preceded by UM represent liver specimens obtained at the University of Michigan. Liver specimens obtained at the Medical College of Wisconsin are represented by the letters of the alphabet. The ages, genders, smoking habits, and drug histories of the patients not previously reported are listed in Table 1.

General assays. Hepatic microsomes were prepared by differential centrifugation, as previously described (19). The protein concentrations of the various microsomal samples and purified P450s were determined colorimetrically (20). Total P450 concentrations were determined by the method of Omura and Sato (21) using the extinction coefficient of 91 mM^{-1} cm^{-1}. SDS-polyacrylamide gel electrophoresis (10 and 12%, w/v) was performed by the method of Laemmli (22) and the separated proteins were silver stained as described by the manufacturer. Ouchterlony double-diffusion immunoprecipitin (23) and immunoblot analyses (4) were carried out as described elsewhere. Peptide maps were generated by limited proteolytic digestion by the method of Cleveland *et al.* (24). Amino-terminal amino acid sequences were determined by automated Edman degradation, using an Applied Biosystems model 477A pulsed liquid phase sequencer.

Purification of human liver cytochrome P-450 HLp3. Unless otherwise indicated, all steps of the purification were carried out at 4°. Liver microsomes (2000 mg of total protein, 1076 nmol of P450) isolated from specimen E were diluted to 2 mg of protein/ml in microsome storage buffer (25) and were solubilized by the dropwise addition of 20% (w/v) twice-recrystallized cholate to a final concentration of 0.6%. The solution was stirred for 30 min and insoluble material was removed by centrifugation for 1 hr at 105,000 × *g*. The resulting supernatant was divided into two equal fractions and applied at 1 ml/min to two aminooctylamino-Sepharose 4B columns (2.6 × 28 cm), which were prepared as previously described (25) and equilibrated in 100 mM potassium phosphate buffer (pH 7.25) that contained 1 mM EDTA, 20% glycerol, and 0.6% cholate. The columns were washed with 1 liter each of equilibration buffer containing 0.42% cholate. Hemoprotein was eluted by equilibration buffer containing 0.33% cholate and 0.06% Tergitol NP-10. The 417 nm-absorbing peak fractions from both columns that were of high relative purity, as determined by silver-stained polyacrylamide gels, and that reacted with a monoclonal anti-HLp antibody (4, 18) in immunoblot analyses were combined (504.9 nmol of P450, 47% yield) and concentrated to 44 ml in an Amicon ultrafiltration cell equipped with a PM30 membrane.

This fraction was then dialyzed against 1 liter of 5 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 20% glycerol, 0.1% Tergitol NP-10, and 0.2% cholate over 72 hr with six changes. The dialyzed fraction was then stirred at room temperature for 30 min and applied to a DE51 (1.6 × 7 cm), DE52 (1.6 × 15 cm) and DE53 (1.6 × 25 m) cellulose column series that was equilibrated at 40 ml/hr at room temperature with 1000 ml of dialysis buffer. The columns were then washed with dialysis buffer, which resulted in the elution of HLp3, as determined by immunoblots developed with the monoclonal anti-HLp antibody. Those fractions that were of high relative purity, as determined by SDS-polyacrylamide gel electrophoresis, and were enriched for HLp3 over HLp, as determined by immunoblots, were combined (47.7 nmol of P450, 4.4% yield).

The combined fractions were dialyzed against 20 volumes of 10 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.3% Tergitol NP-10, 1 mM EDTA, and 0.1 mM dithiothreitol for 20 hr. This fraction was then applied to a hydroxylapatite column (1.6 × 5 cm) that was equilibrated at 1 ml/min in 100 ml of the dialysis buffer. The column was washed with 60 ml of 40 mM potassium phosphate buffer (pH 7.25) containing the same components as the dialysis buffer and HLp3 was eluted with 90 ml of 90 mM potassium phosphate buffer with the same components. Those fractions that contained HLp3 and that were of high relative purity were combined (16.0 nmol of P450, 1.5% yield).

The combined fractions were then dialyzed against 20 volumes of 5 mM potassium phosphate buffer (pH 6.5) containing 20% glycerol, 0.1 mM EDTA, and 0.2% Tergitol NP-10, with four changes over 48 hr. The dialyzed fraction was applied at 1 ml/min to a CM52 column (1.6 × 10 cm) that was equilibrated with 200 ml of dialysis buffer. The column was washed with 120 ml of dialysis buffer and the hemoprotein was eluted by application of a 200-ml 0–250 mM NaCl gradient in dialysis buffer. Only one 417 nm-absorbing peak eluted and those fractions within that peak that contained only HLp3, as determined by immunoblots and SDS-polyacrylamide gel electrophoresis, were combined. A small hydroxylapatite column (26) was used to concentrate and remove the detergent from HLp3 (3.15 nmol of P450, 0.3% final yield).

Purification of other proteins and antibody preparation. Adult human liver HLp was purified to a specific content of 17.5 nmol of P450/mg of protein, by previously described methods (4), from microsomes isolated from specimen 11. Human fetal liver HLp2 was purified as previously described (6). Rat NADPH cytochrome P-450 reductase was purified as previously described by Yasukochi and Masters (27). Monoclonal antibody 13-7-10, which recognizes HLp (4, 18, 28), and goat anti-HLp IgG (6) were prepared and characterized as indicated in the cited references.

Testosterone hydroxylase assay. The rates of testosterone hydroxylation by liver microsomes or by reconstituted systems were determined by a slight modification of the procedure described by Sonderfan *et al.* (29). Briefly, liver microsomes (0.25 to 0.60 nmol of P450) or reconstituted systems (0.05 nmol of P450 reconstituted with optimal amounts of NADPH cytochrome P450 reductase and dilauroylphosphatidylcholine, 0.2 nmol and 10 μg, respectively) were incubated at 37° in 1-ml duplicate incubation mixtures that contained 0.25 mM testosterone, 3 mM $MgCl_2$, 1 mM EDTA, and 50 mM potassium phosphate buffer (pH 7.4). Reactions were initiated by the addition of NADPH (1.2 mM). Under these conditions, the rates of formation of the various testosterone metabolites were linear for at least 10 min. The reaction was terminated with 6 ml of dichloromethane, spiked with internal standard (11β-hydroxytestosterone), vortexed, and centrifuged and 4 ml of the organic phase were dried under nitrogen. The dried samples were stored at −20°. The residue was redissolved in 50

TABLE 1

Patient histories and immunoquantification of total HLp-related protein and HLp3 levels

Immunoquantification of the human liver P450s related to HLp were performed as described in Experimental Procedures, with the densitometric value obtained for specimen A arbitrarily set at 100%.

Patient Code	Total HLp-Related[a]	HLp3	Gender	Age	Smoking Habits[b]	Drug History
	%	% of total			pack-years	
A	100		M	25	75	None
B	110		M	50	35	None
C	118		M	22	UK[c]	Ethanol (0.25%) (unspecified drug abuse)
D	73		M	31	NS[d]	None
E	262	26	M	14	NS	Pentobarbital (coma induced 1 week before death)
						Pancuronium Br
						Dopamine
						Furosemide
						Mannitol
						Heparin
						Cefazolin
F	192	28	F	50	Heavy	Alcoholic
						Insulin
G	218	32	F	48	UK	Teldrin
H	73		F	28	UK	None
I	316		M	43	NS	Phenobarbital
						Phenytoin
						Propranolol
J	121		F	55	UK	None
24	41		UK	UK	UK	UK
25	61		M	27	UK	Dopamine
						Vasopressin
26	48		F	69	NS	None
27	40		M	47	UK	Lorazepam
						Cefazolin
						Triazolam
						Morphine
						Glycopyrrolate
28	80		F	75	NS	Levothyroxine
						Thioridazine
						Furosemide
						Ranitidine
29	81		F	66	75	Levothyroxine
						Estrogen
						Hydroxyzine
						Metamucil
30	212	15	F	40	NS	$FeSO_4$
						Cefoxitin
31	122		F	57	NS	Furosemide
						Morphine
						Promethazine
						Scopolamine
						Codeine
						Milk of magnesia
						Diphenhydramine
32	178		M	44	NS	Triamterene
						Hydrochlorothiazide
33	212		F	67	50	Theophylline
34	291	24	M	52	40	None
35	204	18	F	67	NS	None
UM-4	143		F	60	NS	Ativan
						Cefoxitin
UM-5	139		F	48	50	Furosemide
						Ranitidine
						Spironolactone
						Chlordiazepoxide
						Diphenhydramine
UM-11	129	21	M	30	UK	Dopamine
						Heparin
UM-13	72		M	26	NS	Cyclosporine A
						Prednisone
						Azathiaprine
UM-14	113		UK	UK	UK	UK

[a] Immunoquantification of total HLp-related protein in the microsomal sample. In addition, the 100% value equals 0.058 nmol of HLp/mg of microsomal protein, as determined by comparison of the densitometric values obtained for specimen A with a standard curve generated with purified HLp.

[b] Values denotes pack-years, which are defined as the number of packages of cigarettes per day × years smoked.

[c] UK, unknown.

[d] NS, nonsmoker.

μl of solvent A (methanol/water/acetonitrile, 39:60:1) and sonicated in a bath sonicator for 15 min. Testosterone and its metabolites were resolved using a Beckman model 332 gradient HPLC system equipped with a Supelcosil LC-18 (5 μm, 15 × 4.6 mm; Supelco, Bellefonte, PA) column preceded by a 2-cm LC-18 guard column (40 μm; Supelco). A 23-min concave gradient, similar to that used by Sonderfan *et al.* (29), of 90% solvent A to 100% solvent B (methanol/water/acetonitrile, 80:18:2) was applied at 1.5 ml/min to the column to elute testosterone and its metabolites, which were detected by a Spectra-Physics model 770 variable wavelength UV detector set at 254 nm. Metabolites were quantified by comparisons of their peak areas (determined using a Hewlett-Packard model 3390A integrator) to those of authentic standards, which were graciously supplied by Dr. Andrew Parkinson. In addition, an unidentified metabolite eluted between 16 β and 18-hydroxytestosterone at approximately 18 min.

The immunoinhibition studies were performed, as previously described (4), on incubations containing microsomes isolated from specimen A, using a ratio of 20 mg of anti-HLp IgG to 1 nmol of total P450.

Results

Identification of HLp3. Immunoblots of human liver microsomes developed with either a polyclonal or monoclonal antibody that recognizes HLp have demonstrated that all adult human liver specimens previously examined contain a high amount of a P450 that migrates in SDS-polyacrylamide gels with the same molecular weight as HLp (4, 6, 9). However, as shown in Fig. 1, we recently obtained a human liver specimen that clearly contains two proteins immunochemically related to HLp. The faster migrating protein is indistinguishable from HLp on immunoblots developed with the monoclonal anti-HLP antibody (Fig. 1A). In addition, the slower migrating protein (here termed HLp3) in the specimen does not comigrate with HLp2, a form of P450 highly related to the HLp (6), in fetal liver microsomes (Figure 1B). These data suggest that HLp3 represents a new member of the P450III family. A close examination of our bank of human liver microsomes for other specimens containing HLp3 revealed that 7 of 27 human liver specimens contained detectable levels of HLp3. The concentration of HLp3 expressed as a percentage of the total amount of anti-HLp-reactive protein in microsomes isolated from these liver specimens is shown in Table 1. In addition, upon reexamination of the 19 liver samples (specimens 1–4, 6–12, and 16–23) previously reported by us to contain HLp (9), 4 were found to contain detectable levels of HLp3. Those liver specimens were numbers 7, 9, 10, and 20, in which HLp3 was found to be 26, 19, 14, and 15%, respectively, of the total HLp-related protein. As previously reported (9), only patient 9 of these 4 patients had received known inducers of the P450III family (dexamethasone and phenytoin). Thus, HLp3 was detected in 11 of a total of 46 specimens and the amount of HLp3 in these 11 specimens varied between 14 and 32% of the total HLp-related protein. The levels of HLp3 did not correlate with the gender, age or smoking habits of the patient. Of the 11 patients expressing HLp3, only patients E and 9 had received known inducers of the P450III family. Thus, the expression of HLp3 does not appear to influenced by the drug history of the patients.

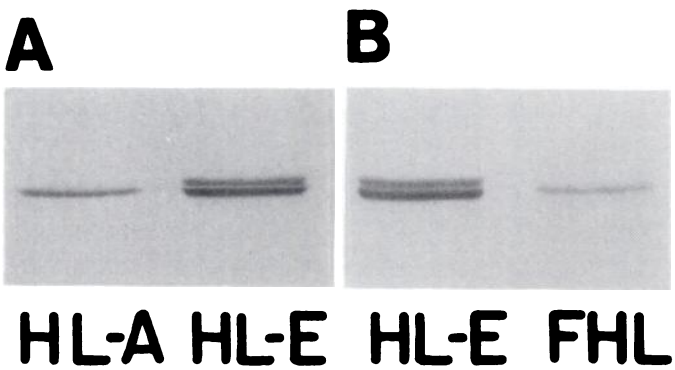

Fig. 1. Immunoblot of human liver microsomes developed with monoclonal anti-HLp. Immunoblot analyses were performed and developed with monoclonal anti-HLp antibody as described in Experimental Procedures, using microsomes isolated from human livers A (20 μg) or E (20 μg) (A) or liver E (20 μg) or a fetal liver specimen (*FHL*; 35 μg) (B).

Purification of HLp3. In order to purify HLp3, cholate-solubilized microsomes from specimen E were subjected to chromatography on aminooctylamino-Sepharose 4B, DEAE-cellulose, hydroxylapatite, and CM-cellulose columns. The various column fractions generated by these procedures that had 417 nm-absorbing material were monitored by immunoblots developed with the monoclonal antibody that recognizes both HLp and HLp3. At each step of the purification procedure, those fractions that were enriched for HLp3 over HLp were combined. This procedure resulted in a poor overall yield of HLp3 (final yield, 0.3%) but resulted in a preparation of HLp3 that was devoid of all other proteins, most importantly HLp, as determined by silver-stained SDS-polyacrylamide gels (Fig. 2A) and immunoblots developed with the anti-HLp monoclonal antibody (Fig. 2B).

The elution profile of HLp3 from the aminooctylamino-Sepharose 4B and CM-cellulose columns was similar to that observed for the elution of HLp (4) and HLp2 (6) from these columns. However, HLp3 did not tightly bind the DEAE-cellulose series of columns, which is unlike that observed for HLp (4) but is similar to what was observed for HLp2 (6). The final preparation of HLp3 was homogeneous as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2A), and had a specific content of 8.1 nmol of P450/mg of protein. Spectral analyses (not shown) indicated that HLp3 exists primarily in the low spin state and has a Soret maximum at 448.5 nm in the CO-reduced state, which is identical to HLp but is different from HLp2 (449.5) (6).

Characterization of HLp3. The apparent monomeric molecular weight of HLp3 is 52 kDa, as determined by its migration in an SDS-polyacrylamide gel, which is slightly greater than that of either HLp (51 kDa) or HLp2 (51.5 kDa) (6). Fig. 3, *lanes 1–3*, demonstrates that these three proteins, detected by silver staining or immunoblotting, migrate in SDS-polyacrylamide gels (10%) at slightly different rates. In addition, in mixtures, HLp and HLp3 or HLp2 were clearly separated (Fig. 3, *lanes 4* and *6*). However, mixtures of HLp2 and HLp3 did not resolve (Fig. 3, *lane 5*), despite the fact that they migrate at slightly different rates as pure proteins (Fig. 3, *lanes 2* and *3*) and in microsomal samples, as determined by immunoblots developed with an antibody to HLp (Fig. 1B).

Because HLp3 was purified by monitoring the fractions that were generated by the various procedures with immunoblots developed with a monoclonal antibody that also recognizes HLp and HLp2 (Fig. 2B), HLp3 is immunochemically related to the P450III family. In addition, HLp3 was also recognized on immunoblots by polyclonal antibodies against HLp, rat P450p, and rabbit LM3c (data not shown). Ouchterlony double-diffusion analyses with goat polyclonal anti-HLp IgG were used to

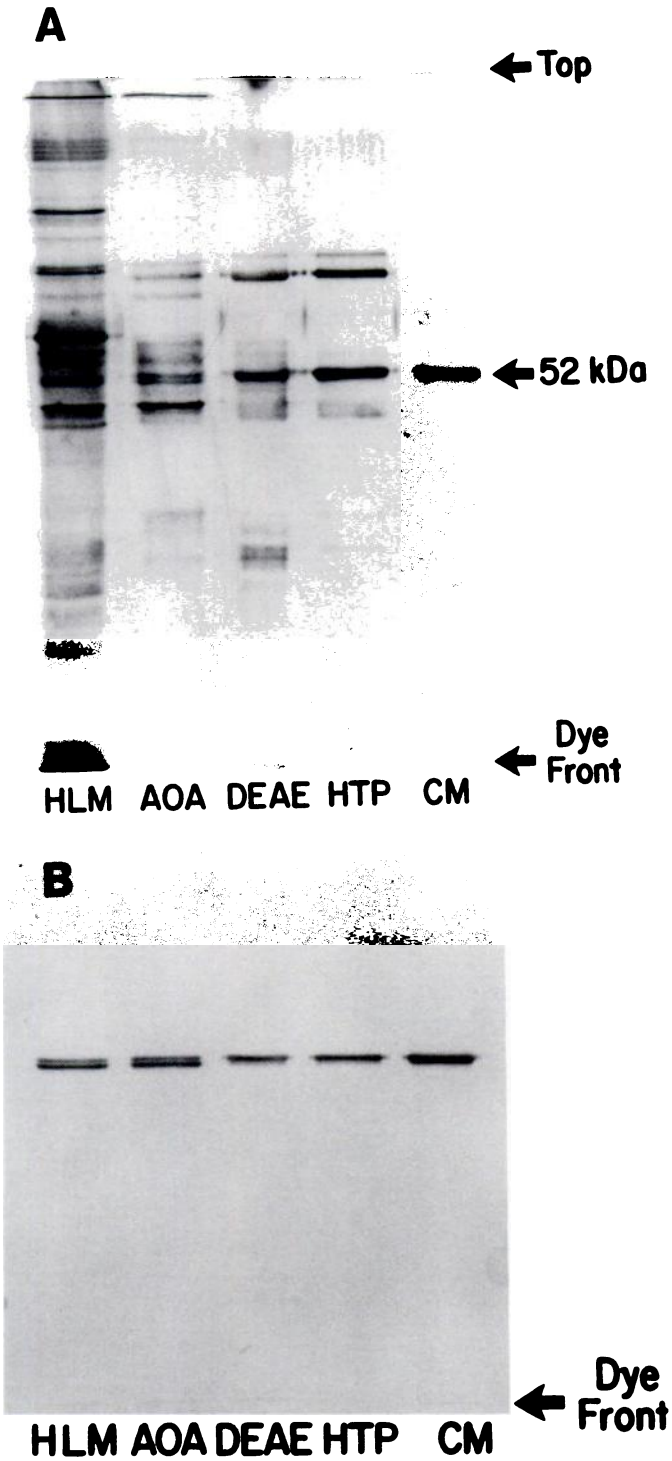

Fig. 2. Electrophoretic profile of the combined fractions obtained at each step of the purification of HLp3. Microsomes from specimen E (*HLM*; 7.5 μg of protein) and the final combined fractions from the aminooctylamino-Sepharose 4B (*AOA*), DEAE-cellulose series (*DEAE*), hydroxylapatite (*HTP*), and CM52 (*CM*; 1 μg of protein) cellulose columns were subjected, as described in Experimental Procedures, to electrophoresis in a SDS-polyacrylamide gel (10%) and the proteins were visualized by silver staining (A) or immunoblotting with the monoclonal anti-HLp antibody (B).

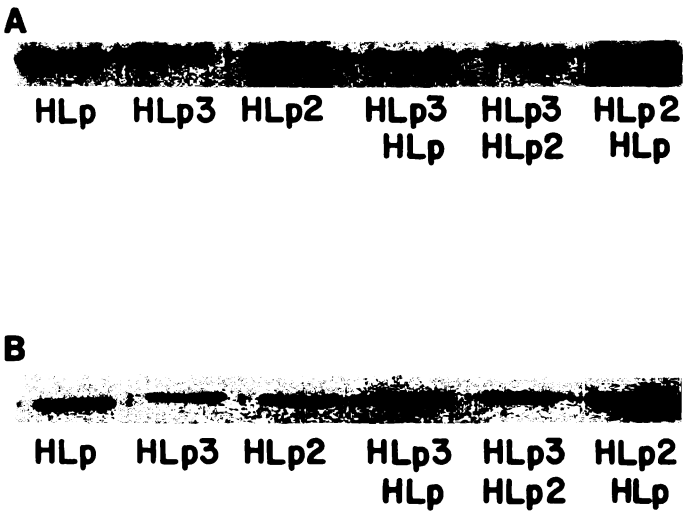

Fig. 3. Electrophoretic profile of purified HLp, HLp2, and HLp3. The purified proteins were prepared, subjected to electrophoresis in SDS-polyacrylamide gels (10%), and visualized, as described in Experimental Procedures, by silver staining (A) or immunoblotting with the monoclonal anti-HLp antibody (B) as individual proteins (0.5 μg each for silver staining and 0.25 μg each for immunoblotting) or as mixtures of two (0.25 μg each for silver staining and 0.125 μg each for immunoblotting).

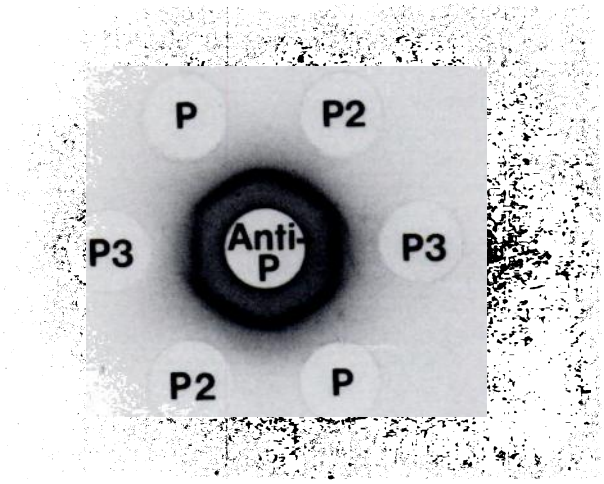

Fig. 4. Ouchterlony double-diffusion analysis with HLp, HLp2, and HLp3, using goat polyclonal anti-HLp IgG. The center well was filled with goat anti-HLp IgG (*Anti-P*) and the outside wells were filled with buffer containing 15 μg/ml HLp (*P*), HLp2 (*P2*), or HLp3 (*P3*). The analysis was preformed as described in Experimental Procedures.

further characterize the immunochemical relationships between HLp3, HLp2, and HLp (Fig. 4). As previously observed with this antibody (6), HLp and HLp2 both form strong immunoprecipitin bands that fuse to form a line of identity. However, with this antibody HLp3 forms only a weak immunoprecipitin band that fuses with the bands formed with HLp and HLp2, creating lines of partial identity. The spurs extending past the junction of the HLp and HLp2 bands with the HLp3 band indicate that some of the antigenic determinants present in HLp and HLp2 are not present in HLp3.

Amino-terminal amino acid sequence analysis has been demonstrated to be useful in the characterization of even closely related P450s (2). Automated Edman degradation of HLp3 yielded a 28-amino acid sequence (Table 2). In comparison with other HLp-related human P450s, the sequence obtained from

TABLE 2

Amino-terminal amino acid sequences of HLp3 and related P450s

The sequences for HLp2 (6), HLp (4), P450p (34), PCN2 (33), PCNc (34), and LM3c (2) are given for comparison and underlined amino acids differ from that determined for HLp3.

Species	Form	Residues identified																										Matches with HLp3
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Human	HLp3	M	D	L	I	P	N	L	A	V	E	T	X	L	L	L	A	V	S	L	V	L	L	Y	L	Y	G	T	R	T	28/28
	HLp2	M	D	L	I	P	N	L	A	V	E	T	X	L	L	L	A	V	S	L	I	L	L	Y	L	Y	X	T	R	T	26/27
	HLp	M	A	L	I	P	D	L	A	M	Q	T	W	L	L	L	A	V	S	L	V	L	L	Y	L	Y	G	T	H	S	22/28
Rat	P450p	M	D	L	L	S	A	L	T	L	E	T	W	V	L	L	A	V	V	L	V	L	L	Y	G	F	G	T	R	T	19/28
	PCN2	M	D	L	L	S	A	L	T	L	E	T	W	V	L	L	A	V	I	L	V	L	L	Y	R	L	G	T	H	R	17/28
	PCNc	M	D	L	L	S	A	L	T	L	E	T	W	V	L	L	A	V	I	L	V	L	L	Y	R	L	G	T	H		17/22
Rabbit	LM3c	M	D	L	I	F	S	L	E	T	W	V	L	L	A	A	S	L	V	L	L	Y	L	Y							9/22

HLp3 shares 79 and 96% homology with, respectively, HLp and HLp2. Furthermore, the sequence obtained for HLp3 is no more than 30% homologous with that reported for six other human P450s (9, 11). Finally, with respect to amino-terminal amino acid sequences reported for rat and rabbit P450s, the sequence for HLp3 is highly similar to P450III family members isolated in those species (i.e., rat P450p, 68%; rat PCN2, 61% rat PCNc, 63%; and rabbit LM3c, 41%) but was no more than 36% similar to the sequences reported for 14 other rat or rabbit P450s (2).

Peptide mapping by limited proteolytic digestion of proteins has also been used to determine whether isolated proteins are structurally similar. Peptide maps of HLp, HLp2, and HLp3 generated by limited digestion with *S. aureus* V8 and α-chymotrypsin are shown in Fig. 5. The SDS-polyacrylamide gel electrophoretic profiles of the peptides generated from the three proteins with both proteases, detected by silver staining (Fig. 5A) or immunoblotting with the monoclonal antibody to HLp (Fig. 5B), yielded maps for HLp, HLp2, and HLp3 that have some similarities but are clearly distinct. Therefore, these three proteins have distinct primary structures.

Catalytic properties. The *in vitro* stereospecific formation of metabolites of testosterone has been used extensively to profile the P450s present in rat liver microsomal samples (29). However, in a recent report using thin layer chromatography for the separation of the metabolites of testosterone, a comparatively few metabolites were formed by human liver microsomes (only 6β-, 2β-, and 15β-hydroxytestosterone and androstenedione were formed) (16). As shown in Table 3, using a highly sensitive HPLC method developed by Sonderfan *et al.* (29) for the separation of the testosterone metabolites, we were able to detect not only those metabolites previously observed but also 6α-, 16α-, 16β-, and 18-hydroxytestosterone and an unidentified metabolite of testosterone. The only metabolites of testosterone formed by rat liver microsomes that were not detected in incubations with human liver microsomes were 1α/β-, 2α-, and 7α-hydroxytestosterone.

In order to determine whether HLp3 catalyzes unique stereospecific hydroxylations of testosterone, we compared the metabolism of testosterone by liver microsomes isolated from three patients who expressed HLp3 (patients E, F, and G) and seven patients (A–D and H–J) who did not express HLp3. A previous study determined that, in human liver microsomes, the levels of P450NF (HLp)-related protein quantified by immunoblot analyses correlated well with the *in vitro* rates of formation of 6β-, 2β-, and 15β-hydroxytestosterone (16). In our studies, not only did these three hydroxylations correlate with total HLp-related protein levels ($r \geq 0.85$), but so did the rates of formation of 6α-hydroxytestosterone ($r = 0.79$), 18-hydroxytestosterone ($r = 0.83$), 16β-hydroxytestosterone ($r = 0.79$), and an unidentified metabolite ($r = 0.85$). Only the rates of formation of 16α-hydroxytestosterone and androstenedione did not correlate with HLp levels. Furthermore, preincubation of microsomes isolated from specimen A with the polyclonal anti-HLp IgG inhibited the production of 6α-, 6β-, 15β-, and 18-hydroxytestosterone and the unknown (≥70%) while having little

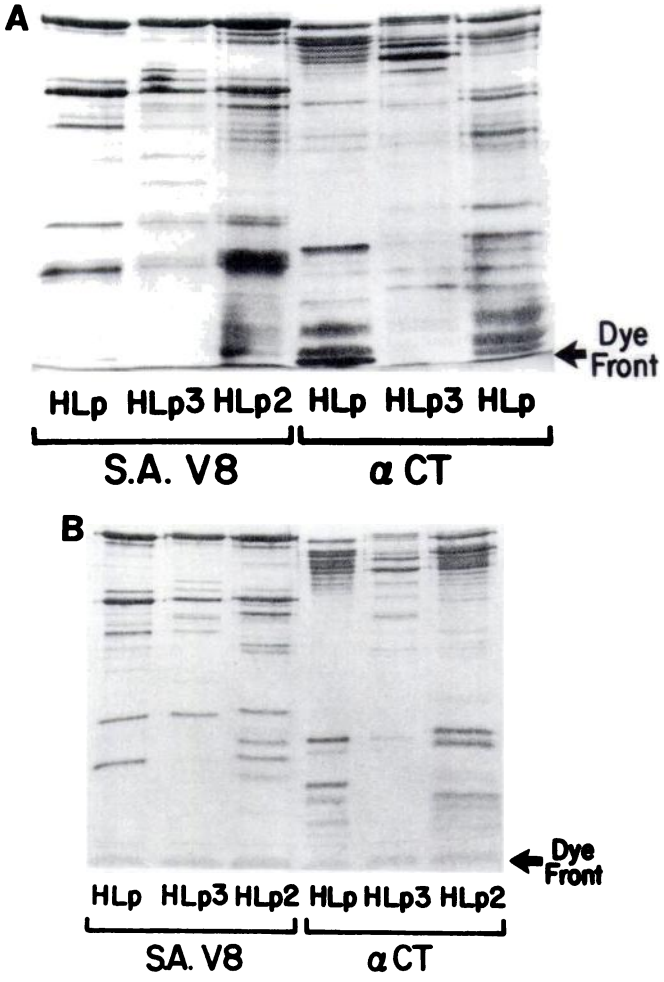

Fig. 5. Peptide maps of HLp, HLp2, and HLp3. The P450s were digested with *S. aureus* V8 (*S.A. V8*) or α-chymotrypsin (α-*CT*) and the peptides were separated by SDS-polyacrylamide gel (12%) electrophoresis as previously described (6). The peptides were visualized by either silver staining (A) or immunoblotting with monoclonal anti-HLp antibody (B).

TABLE 3

Testosterone hydroxylase activities of human liver microsomes

Testosterone hydroxylase activities were determined using human liver microsomes as described in Experimental Procedures.

Specimen	Testosterone Metabolism									
	2βOH[a]	6αOH	6βOH	15βOH	16αOH	16βOH	18	A	Total	Unknown[b]
	pmol of product/min/mg									
A	102.6 (1.0)[c]	0.9 (1.0)	1693.5 (1.0)	58.0 (1.0)	6.4 (1.0)	23.4 (1.0)	6.8 (1.0)	120.6 (1.0)	2011.8 (1.0)	101 (1.0)
B	129.8 (1.3)	2.9 (3.2)	1903.4 (1.1)	62.7 (1.1)	11.2 (1.8)	32.6 (1.4)	8.8 (1.3)	106.9 (0.9)	2258.3 (1.1)	148 (1.5)
C	75.0 (0.7)	1.5 (1.7)	1078.6 (0.6)	33.5 (0.6)	6.8 (1.1)	14.8 (0.6)	3.7 (0.5)	120.8 (1.0)	1334.7 (0.7)	67 (0.7)
D	40.3 (0.4)	0.6 (0.7)	558.2 (0.3)	24.0 (0.4)	17.6 (2.8)	20.4 (0.9)	2.1 (0.3)	100.3 (0.8)	763.5 (0.4)	33 (0.3)
E[d]	425.0 (4.1)	4.8 (5.3)	6122.7 (3.6)	179.5 (3.1)	23.1 (3.6)	77.8 (3.3)	19.9 (2.9)	131.4 (1.1)	6984.2 (3.5)	440 (4.4)
F[d]	198.5 (1.9)	2.5 (2.8)	2946.9 (1.7)	92.9 (1.6)	5.9 (0.9)	31.2 (1.3)	8.4 (1.2)	104.6 (0.9)	3390.9 (1.7)	163 (1.6)
G[d]	104.3 (1.0)	0.6 (0.7)	1528.6 (0.9)	47.2 (0.8)	4.8 (0.8)	22.7 (1.0)	6.2 (0.9)	100.6 (0.8)	1815.0 (0.9)	98 (0.9)
H	132.9 (1.3)	1.3 (1.4)	1946.7 (1.1)	65.5 (1.1)	3.7 (0.6)	33.0 (1.4)	6.6 (1.0)	108.7 (0.9)	2298.4 (1.1)	121 (1.2)
I	536.3 (5.2)	6.0 (6.7)	8406.1 (5.0)	266.2 (4.6)	10.6 (1.7)	80.7 (3.4)	36.7 (5.4)	94.6 (0.8)	9426.6 (4.7)	694 (6.9)
J	160.0 (1.6)	0.9 (1.0)	2375.1 (1.4)	78.0 (1.3)	4.3 (0.7)	32.2 (1.4)	8.9 (1.3)	98.7 (0.8)	2758.1 (1.4)	168 (1.7)

[a] Abbreviations used to denote the position of the hydroxytestosterone formed, except A, which denotes androstenedione.

[b] An unidentified metabolite was formed that was quantified as arbitrary integrator units normalized by comparison with the internal standard and for the amount of microsomal protein and time of incubation.

[c] The numbers in parentheses represent the fold difference in the rate of product formation compared to that determined for specimen A.

[d] Specimens contain both HLp3 and HLp.

effect on the production of 16α-hydroxytestosterone and androstenedione (<12%). The immunoinhibition of the formation of 16β-hydroxytestosterone was less dramatic (about 50%), suggesting that, in addition to HLp-related forms, other P450s may contribute to its production. However, there was no clear correlation between the expression of HLp3 and the *in vitro* production of any testosterone metabolite (Tables 1 and 3).

To determine whether HLp3 is capable of hydroxylating testosterone *in vitro*, we performed the testosterone hydroxylase assay using reconstituted systems containing HLp3 and, for comparison, HLp. In the reconstituted systems with HLp and HLp3, testosterone was hydroxylated primarily at the 6β-position with some metabolism, between 5 and 7% of the total with HLp, at the 2β-position (Table 4). Because cytochrome b_5 has been shown to stimulate the activity of some reconstituted P450 systems (5, 17), cytochrome b_5 was added to reconstituted systems containing HLp and HLp3 and was found to stimulate these systems for the hydroxylation of testosterone by 2.3- and 2.7-fold, respectively (Table 4). In agreement with previous reports on the catalytic activities of P450III family members in reconstituted systems (4, 5, 13, 17), the testosterone hydroxylase activities of HLp and HLp3 were low even in the presence of cytochrome b_5. It is important to note that, under the conditions used in these reconstitution studies, we did not detect the formation of the other metabolites that we associated with the P450III family by their correlation with total HLp-related protein levels and the immunoinhibition studies. However, since 6β- and 2β-hydroxytestosterone are the metabolites formed to the greatest extent in the microsomal studies (Table 3), it would appear that we were unable to detect the other metabolites in the reconstituted systems because they were formed at rates below our limit of detection.

TABLE 4

Testosterone hydroxylase activities of reconstituted HLp and HLp3 preparations

Testosterone hydroxylase activities were determined in reconstituted systems, as described in Experimental Procedure. Reconstituted systems contained: 0.05 nmol of P450, 0.2 nmol of NADPH cytochrome P450 reductase, and 10 μg dilauroylphosphatidylcholine.

Reconstituted System	Testosterone Metabolism		
	6βOH[a]	2βOH	Total activity
	pmol of product/min/nmol of P450		
HLp	318	24	342
HLp + b_5[b]	732	54	786
HLp3	105	ND[c]	105
HLp3 + b_5[b]	270	13	283

[a] Abbreviations used: 6βOH, 6β-hydroxytestosterone; 2βOH, 2β-hydroxytestosterone.

[b] Reconstituted system containing 0.05 nmol of cytochrome b_5.

[c] ND, not detected.

Discussion

The data presented in this manuscript clearly demonstrate that we identified and isolated a human liver P450 (termed HLp3) that is a member of the P450III family and is polymorphically expressed. Direct structural, immunochemical, and functional comparisons of HLp3 with other members of the human P450III family, HLp and HLp2, demonstrate that these three proteins are distinct but highly related. First, HLp3 was initially identified in immunoblot analyses developed with an anti-HLp antibody that also recognizes HLp2 (Fig. 1). In our studies, HLp3 was detected on immunoblots as a protein of a slightly greater apparent molecular weight than either HLp or HLp2. Second, HLp3 was purified from human liver microsomes by procedures similar to those used to isolate HLp and HLp2 and by following the elution of HLp3 from the various

columns by immunoblots developed with the anti-HLp antibody. Third, Ouchterlony double-diffusion analyses with a polyclonal anti-HLp antibody showed a clear line of identity between HLp and HLp2. However, only a weak immunoprecipitin band was observed with HLp3 and this band merged with the immunoprecipitin bands for HLp and HLp2 to form lines of partial identity (Fig. 4). This indicates that not all the antigenic determinants shared by HLp and HLp2 are present in HLp3. Fourth, a number of experiments demonstrated structural differences and similarities between these proteins. Specifically, limited proteolysis of these proteins with two different proteases resulted in peptide maps that were similar. However, for each protein distinct fragments were also observed (Fig. 5). In addition, the amino-terminal amino acid sequence obtained for HLp3 was found to be 96% and 78% homologous with HLp2 and HLp, respectively. Furthermore, a recently reported partial sequence of a cloned human P450III gene exactly predicts the amino acid sequence reported here for HLp3 (30). Finally, the three purified proteins migrate in SDS-polyacrylamide gels at slightly different apparent molecular weights (HLp, 51 kDa; HLp2, 51.5 kDa; and HLp3, 52 kDa). Fifth, in reconstituted systems HLp3 and HLp were found to hydroxylate testosterone at the same positions (6β and 2β) that are characteristic of P450III forms isolated from experimental species (Table 4). Thus, the data presented here clearly demonstrate that HLp3 is a member of the human liver P450III family, which has previously been shown to contain HLp (3, 4), P450NF (3, 5), and HLp2 (6).

The regulation of expression of HLp3 appears to be unique among the various characterized forms of P450 in the human P450III family and among related forms in experimental species. That is, HLp3 was detected in only 11 of 46 liver specimens and its levels did not correlate with any of the parameters examined, including the age, gender, smoking habits, and drug history of the patients (Table 1). This is unlike what has been observed for HLp, which has been shown to be induced in humans by steroids (4) and macrolide antibiotics (4, 14) and which may be influenced by gender (31), as are its related forms in experimental species (29). It is interesting to note that both HLp3 and HLp were detected in the only liver specimen obtained from a juvenile (specimen E). Moreover, HLp3 and HLp were detected in a liver specimen obtained at autopsy from a 2-month-old male, the only other specimen obtained from a child.[2] To date, only HLp2 has been detected in fetal liver microsomes (6), including a specimen obtained at autopsy from a mid-gestation (approximately 24 weeks) male fetus.[2] These observations suggest that during human development HLp2 is expressed exclusively in the fetus and that shortly after birth (within 2 months) through adolescence both HLp and HLp3 can be expressed. In addition, by adulthood HLp appears to be universally expressed, whereas there is a polymorphism in the expression of HLp3. Finally, a recent report indicates that a P450 with the same amino-terminal sequence as that of HLp2 is expressed in low levels in at least one adult human liver (32). The developmental pattern of expression for the human P450III family does not compare well with the patterns of development of this family in the experimental species. For example, in the rat, P450III family members do not appear to be present in the fetus (6). In addition, there is a form in the rat P450III family that is expressed only when an inducer is present (33). Furthermore, there is a constitutively expressed male-specific form in the rat P450III family that is not inducible (33). Thus, great differences in the regulation of the expression of the rat and human P450III families have apparently occurred as a result of evolution.

Previous studies have demonstrated that hepatic HLp levels are elevated in patients receiving dexamethasone (4) or the macrolide antibiotics rifampicin (14) and triacetyloleandomycin (4), as compared with the levels found in patients receiving no known inducers of the P450III family. In addition to steroids and macrolide antibiotics, members of the P450III family have been shown to be induced in the rat by treatment with barbiturates (34). However, in the previous reports on the expression of HLp, no liver specimens were obtained from patients that were treated with only a barbiturate (4, 9). In the current study, two patients received barbiturates or what are referred to as "phenobarbital-like" inducers as the only medications that have been reported to induce P450 levels. The first liver specimen (E) was obtained from a 14-year-old male who, due to severe head trauma, was treated with pentobarbital to induce coma. The second sample (I) was from a 43-year-old male who received phenobarbital and the "phenobarbital-like" inducer phenytoin for the last 20 years of his life to control seizures. Both of these specimens were found to have elevated levels of total HLp-related protein, as compared with specimens from those patients not receiving inducers of the P450III family (Table 1). Although HLp3 was detected in a liver specimen from a patient receiving a barbiturate (E) and a specimen from a patient receiving dexamethasone (specimen 9) (9), it was not detected in all or even the majority of the liver specimens from patients (a total of eight) who received drugs known to induce the P450III family. Thus, HLp appears to be induced in humans by glucocorticoids, macrolide antibiotics, and barbiturates, whereas these agents do not appear to influence the expression of HLp3.

Testosterone is hydroxylated *in vitro* by rat hepatic P450s in a manner that is stereospecific and regioselective (29). Thus, the pattern of testosterone metabolites observed after incubation with rat liver microsomes is indicative of the profile of forms of P450 present in that sample. Several previous studies have shown that the metabolism of testosterone by human liver microsomes is less complex than that by rat microsomes (16, 35). Our data clearly show that, by using a sensitive HLPC technique to detect the formation of testosterone metabolites, the pattern of metabolites observed with human liver microsomes is nearly as complex as that obtained with rat microsomes (9 versus 11 detected metabolites). However, unlike what has been observed with rat microsomes, the vast majority of the metabolism of testosterone by human liver microsomes appears to be catalyzed by the P450III family. That is, the inhibition by the anti-HLp antibody of nearly all of the metabolism of testosterone by human liver microsomes and the excellent correlations between HLp-related protein levels and the rates of hydroxylation of testosterone at the 2β, 6α, 6β, 15β, 16β, and 18 positions and the formation of the unidentified metabolite indicate that, in human liver microsomes, the P450III family is responsible for the vast majority of the metabolism of testosterone. Moreover, a human fetal P450 termed HFLa, which appears to be identical to HLp2 (6), has been shown to be responsible for the majority of the metabolism

[2] S. A. Wrighton, unpublished observation.

of testosterone by human fetal liver microsomes (36). These data suggest that, throughout the development of humans, the P450III family plays a pivotal role in the hepatic clearance of testosterone. In addition, it would appear that, like the regulation of the expression of the P450III family, in the course of evolution the substrate specificities of the various P450s have been altered with respect to that observed in the rat, because several different rat forms of P450 are responsible for the hydroxylation of testosterone at many of the positions that are hydroxylated in humans by only HLp-related proteins. The observations reported here indicate that studies on hepatic xenobiotic and endogenous compound metabolism and on the regulation of the expression of the P450s using the rat model should be applied to the human situation with caution.

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References

1. Adesnik, M., and M. Atchison. Genes for cytochrome P-450 and their regulation. *Crit. Rev. Biochem.* **19**:247–305 (1985).
2. Black, S. D., and M. J. Coon. Comparative structures of P-450 cytochromes, in *Cytochrome P-450. Structure, Mechanism and Biochemistry* (P. R. Ortiz de Montellano, ed.). Plenum Publishing Corp., New York, 161–216 (1986).
3. Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P-450 gene superfamily: recommended nomenclature. *DNA* **6**:1–11 (1987).
4. Watkins, P. B., S. A. Wrighton, P. Maurel, E. G. Schuetz, G. Mendez-Picon, G. A. Parker, and P. S. Guzelian. Identification of an inducible form of cytochrome P-450 in human liver. *Proc. Natl. Acad. Sci. USA* **82**:6310–6314 (1985).
5. Guengerich, F. P., M. V. Martin, P. H. Beaune, P. Kremers, T. Wolff, and D. J. Waxman. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **261**:5051–5060 (1986).
6. Wrighton, S. A., and M. VandenBranden. Isolation and characterization of human fetal liver cytochrome P-450HLp2: a third member of the P450III gene family. *Arch. Biochem. Biophys.* **268**:144–151 (1989).
7. Wrighton, S. A., C. Campanile, P. E. Thomas, S. L. Maines, P. B. Watkins, G. Parker, G. Mendez-Picon, M. Haniu, J. E. Shively, W. Levin, and P. S. Guzelian. Identification of a human liver cytochrome P-450 homologous to the major isosafrole-inducible cytochrome P-450 in the rat. *Mol. Pharmacol.* **29**:405–410 (1986).
8. Wrighton, S. A., P. E. Thomas, D. T. Molowa, M. Haniu, J. E. Shively, S. L. Maines, P. B. Watkins, G. Parker, C. Mendez-Picon, W. Levin, and P. S. Guzelian. Characterization of ethanol-inducible human liver *N*-nitrosodimethylamine demethylase. *Biochemistry* **25**:6731–6735 (1986).
9. Wrighton, S. A., P. E. Thomas, P. Willis, S. L. Maines, P. B. Watkins, W. Levin, and P. S. Guzelian. Purification of a human liver cytochrome P-450 immunochemically related to several cytochromes P-450 purified from untreated rats. *J. Clin. Invest.* **80**:1017–1022 (1987).
10. Shimada, T., K. S. Misono, and F. P. Guengerich. Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism: purification and characterization of two similar forms involved in the reaction. *J. Biol. Chem.* **261**:909–921 (1986).
11. Distlerath, L. M., P. E. B. Reilly, M. V. Martin, G. G. Davis, G. R. Wilkinson, and F. P. Guengerich. Purification and characterization of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin *O*-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **260**:9057–9067 (1985).
12. Adams, D. J., S. Seilman, Z. Amelizad, F. Oesch, and R. Wolf. Identification of human cytochromes P-450 analogous to forms induced by phenobarbital and 3-methylcholanthrene in the rat. *Biochem. J.* **232**:869–876 (1985).
13. Gonzalez, F. J., B. J. Schmid, M. Umeno, O. W. McBride, J. P. Hardwick, U. A. Meyer, H. V. Gelboin, and J. R. Idle. Human P450PCN1: sequence, chromosome localization, and direct evidence through cDNA expression that P450PCN1 is nifedipine oxidase. *DNA* **7**:79–86 (1988).
14. Kronbach, T., V. Fischer, and U. A. Meyer. Cyclosporin metabolism in human liver: identification of a cytochrome P450III gene family as the major cyclosporin-metabolizing enzyme explains interactions of cyclosporin with other drugs. *Clin. Pharmacol. Ther.* **43**:630–635 (1988).
15. Guengerich, F. P., D. Muller-Enoch, and I. A. Blair. Oxidation of quinidine by human liver cytochrome P-450. *Mol. Pharmacol.* **30**:287–295 (1986).
16. Waxman, D. J., C. Attisano, F. P. Guengerich, and D. P. Lapenson. Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6β-hydroxylase cytochrome P-450 enzyme. *Arch. Biochem. Biophys.* **263**:424–436 (1988).
17. Kawano, S., T. Kamataki, T. Yasumori, Y. Yamazoe, and R. Kato. Purification of human liver cytochrome P-450 catalyzing testosterone 6β-hydroxylation. *J. Biochem.* **102**:493–501 (1987).
18. Molowa, D. T., E. G. Schuetz, S. A. Wrighton, P. B. Watkins, P. Kremers, G. Mendez-Picon, G. A. Parker, and P. S. Guzelian. Complete cDNA sequence of a cytochrome P-450 inducible by glucocorticoids in human liver. *Proc. Natl. Acad. Sci. USA* **83**:5311–5315 (1986).
19. van der Hoeven, T. A., and M. J. Coon. Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. *J. Biol. Chem.* **249**:6302–6310 (1974).
20. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
21. Omura, T., and R. Sato. The carbon monooxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
22. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
23. Thomas, P. E., L. M. Reik, D. E. Ryan, and W. Levin. Induction of two immunochemically related rat liver cytochrome P-450 isozymes, cytochromes P-450c and P-450d, by structurally diverse xenobiotics. *J. Biol. Chem.* **258**:4590–4598 (1983).
24. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J.Biol. Chem.* **252**:1102–1106 (1977).
25. Wang, P. P., P. Beaune, L. S. Kaminsky, G. A. Dannan, F. F. Kadlubar, D. Larrey, and F. P. Guengerich. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. *Biochemistry* **22**:5375–5383 (1983).
26. Wrighton, S. A., P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young and P. S. Guzelian. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid-responsive cytochrome P-450p. *Biochemistry* **24**:2171–2178 (1985).
27. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
28. Beaune, P., P. Kremers, F. Letawe-Goujon, and J. E. Gielen. Monoclonal antibodies against human liver cytochrome P-450. *Biochem. Pharmacol.* **34**:3547–3552 (1985).
29. Sonderfan, A. J., M. P. Arlotto, D. R. Dutton, S. K. McMillen, and A. Parkinson. Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P-450. *Arch. Biochem. Biophys.* **255**:27–41 (1987).
30. Guzelian, P. S. Regulation of the glucocorticoid-inducible cytochromes P-450, in *Microsomes and Drug Oxidations* (J. O. Miners, D. J. Birkett, R. Drew, B. K. May, and M. E. McManus, eds.). Taylor and Francis, New York,148–155 (1988).
31. Watkins, P. B., S. A. Murray, L. G. Winkelman, D. M. Heuman, S. A. Wrighton, and P. S. Guzelian. The erythromycin breath test as an assay of glucocorticoid-inducible liver cytochromes P-450: studies in rats and patients. *J. Clin. Invest.* **83**:688–697 (1989).
32. Komori, M., T. Hashizume, H. Ohi, T. Miura, M. Kitada, K. Nagashima, and T. Kamataki. Cytochrome P-450 in human liver microsomes: high-performance liquid chromatographic isolation of three forms and their characterization. *J. Biochem.* **104**:912–916 (1988).
33. Gonzalez, F. J., B.-J. Song, and J. P. Hardwick. Pregnenolone 16α-carbonitrile-inducible P-450 gene family: gene conversion and differential regulation. *Mol. Cell. Biol.* **6**:2969–2976 (1986).
34. Halpert, J. R. Multiplicity of steroid-inducible cytochromes P-450 in rat liver microsomes. *Arch. Biochem. Biophys.* **263**:59–68 (1988).
35. Kremers, P., P. Beaune, T. Cresteil, J. DeGraeve, S. Columelli, J.-P. Leroux, and J. E. Gielen. Cytochrome P-450 monooxygenase activities in human and rat liver microsomes. *Eur. J. Biochem.* **118**:599–606 (1981).
36. Kitada, M., T. Kamataki, K. Itahashi, T. Rikihisa, and Y. Kanakubo. Significance of cytochrome P-450 (P-450 HLFa) of human fetal livers in the steroid and drug oxidations. *Biochem. Pharmacol.* **36**:453–456 (1987).

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